PUTRESCINE BIOSYNTHESIS IN MAMMALIAL CELLS: ESSENTIAL FOR DNA SYNTHESIS BUT NOT FOR MITOSIS

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SUMMARY: The object of this study was to examine the role of putrescine in the regulation of DNA synthesis and mitosis in synchronized Chinese hamster ovary cells using 1,3-diaminopropane (DAP) which is a potent inhibitor of ornithine decarboxylase (EC 4.1.17). Inhibition of putrescine biosynthesis significantly reduced the incorporation of [^{3}H]-TdR into DNA but had no effect on the progression of cells from G1 to S phase. However, inhibition of putrescine synthesis in synchronized S phase cells did not affect their progression to mitosis. In these experiments, the DAP treatment had little or no effect on the levels of spermidine and spermine. These results indicate that putrescine biosynthesis is essential for the completion of DNA synthesis but not required for mitosis and cell division.

INTRODUCTION

The polyamines, particularly, putrescine, spermidine and spermine have been shown to have a role in the proliferation of mammalian cells. The stimulation of cell cultures by the addition of serum or insulin to the medium (1,2,3), partial hepatectomy of rat livers (4,5) and infection of cells with tumorigenic viruses (6,7,8) lead to increased levels of polyamine synthesis. However, the exact role of polyamines in cell proliferation remains to be elucidated. Using specific inhibitors of polyamine biosynthesis, viz., methylglyoxal-bis (guanylhydrazone) (9) and α-methylornithine (10), it was shown of all the polyamines spermidine plays a vital role in the regulation of DNA synthesis and cell division (11). Studies from other laboratories suggest that putrescine may also play an important role in DNA synthesis and mitosis (12).

Abbreviations:-DAP, 1,3-diaminopropane; ODC, L-ornithine decarboxylase; CHO, Chinese hamster ovary; G1, Pre-DNA synthetic period; S, DNA synthetic period; PCC, prematurely condensed chromosomes; G2, Post-DNA synthetic period.

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In our study, we used 1,3-diaminopropane (DAP), the most potent inhibitor of L-ornithine decarboxylase (ODC) available (13), to reexamine the role of putrescine in DNA synthesis and mitosis in Chinese hamster ovary cells. Our results indicate that putrescine biosynthesis is essential for the completion of DNA synthesis but is not required for mitosis and cell division.

MATERIALS AND METHODS

Cells and Cell Synchrony

Chinese hamster ovary (CHO) cells were routinely grown as monolayer cultures as described earlier (16). These cells have a cell cycle time of 12.5 hr and the durations of G1, S, G2 and mitotic periods are 4.0, 5.5, 2.0 and 1.0 hr, respectively. CHO cells were synchronized in S phase by double TdR block method (4). A pulse labeling of these cells with [^{3}H]-TdR indicated that about 96% were in S phase. To obtain mitotic cells, an exponentially growing culture was partially synchronized by a single TdR block which was followed by a colcemid block for 6 hr and the separation of mitotic cells by selective detachment. Cells thus harvested had a mitotic index of about 98%.

Cell Kinetics

In this study, it was essential to determine the optimal effective dose of 1,3-diaminopropane (DAP) where the growth inhibition was maximum without obvious external cell damage or lysis. Stock solution (10 mg/ml) of DAP was prepared freshly, filter sterilized and the desired concentrations were obtained by serial dilution with complete culture medium. Cultures in exponential growth were treated with various concentrations of DAP. After 16 hr (approx. one cycle time) of incubation with DAP, colcemid (0.05 μg/ml) was added and incubation continued for another 8 hr in order to measure the progression of cells into mitosis. The experiment was terminated by trypsinizing the cells and depositing them on slides using a cytocentrifuge. These cells were fixed in methanol-glacial acetic acid (3:1), stained with acetoorcein and scored for the percent of cells in mitosis.

To answer the question as to where the DAP treated cells were blocked, we applied the phenomenon of premature chromosome condensation (15). This method, which involves the Sendai virus - mediated fusion between mitotic and interphase cells, makes it possible to determine the position of an interphase cell in the cell cycle, on the basis of the morphology of its prematurely condensed chromosomes (PCC). Recently, this method has been applied for cell cycle analysis (16,17). CHO cells in exponential growth were treated with DAP for 16 hr. At the end of the incubation period, the treated cells were fused with a synchronized population of mitotic CHO cells to induce PCC, chromosome preparations were made and scored for the frequency of the various types of PCC, i.e., G1, S and G2.

Measurement of DNA Synthesis and Polyamine Levels

The experimental protocol includes the collection of synchronized mitotic cells and plating them in 20 (100 mm) Falcon plastic dishes at 3 x 10^6 cells/dish. They were divided into 4 sets (A,B,C and D) of 5 dishes each. Immediately after plating, DAP (13.5 mM) was added to sets A and B while C and D served as controls. To monitor DNA synthesis, [^{3}H]-TdR (1.0 μCi/ml; Sp. act. 6.7 Ci/mmol) was added to sets A and C. The extent of DNA synthesis was mea-

TABLE 1

CELL CYCLE ANALYSIS OF RANDOM AND DAP

TREATED CHO CELLS BY PCC METHOD

	Relative Frequency (%) of Cells In		
	G1	S	G2
Control (Random population)	36	52	12
Treatment (DAP for 16 hr)	1.9	91.4	6.7

sured both by labeling index and the total amount of label incorporated into TCA-precipitable material (18). Sets A and C were used for the estimation of DNA synthesis while sets B and D were used for the determination of polyamine levels.

For determining the polyamine levels, the cell pellet was resuspended in 0.5 ml of 4% sulfosalisylic acid and sonicated using a Branson sonifier (19). The homogenate was then centrifuged at 10,000 x g in a Sorvall RC2B centrifuge. The supernatant so obtained was used to estimate the polyamine levels in a Durrum amino acid analyzer according to Marton and Lee (20). The results presented represent the average of at least two estimates. The coefficient of variability for the estimation of polyamines by our method is less than 4%.

Radioautography

For the determination of labeling indices, cells were incubated with [^{3}H]-TdR (0.1 µCi/ml) for 30 minutes, trypsinized and deposited on clean slides by using a cytocentrifuge. The cells were fixed, extracted three times (for 20 min each) in cold 5% trichloroacetic acid, and processed for radioautography.

RESULTS

Effect of DAP on Cell Cycle Progression

Initially we found that DAP at a concentration of 13.5 mM caused maximum growth inhibition without any obvious cell damage or lysis. The effects of a 16 hr DAP treatment on the cell cycle kinetics of CHO cells are shown in Table 1. A significant decrease in the relative frequency of G1-PCC and a corre-

TABLE 2

EFFECT OF DAP TREATMENT ON POLYAMINE LEVELS
IN CHO CELLS DURING THEIR TRAVERSE
FROM MITOSIS TO S PHASE

Hours after reversal of mitotic block	Control			DAP Treatment		
	n mole/6 x 10^6 cells			n mole/6 x 10^6 cells		
	Putrescine	Spermidine	Spermine	Putrescine	Spermidine	Spermine
1	0.34	6.56	6.12	0*	5.83	5.59
3	1.27	5.82	5.98	0	5.66	6.83
4	1.98	6.99	6.92	0	4.95	6.28
5	0.53	5.19	6.88	0	4.74	6.47
6	0.10	5.57	5.78	0	4.61	6.84

*Less than the sensitivity of amino acid analyser (below 0.02 n moles).

sponding increase in the S-PCC indicated that DAP had no effect on the progression of cells from G1 to S but these cells however, failed to complete S phase. At the end of 16 hr-treatment, most of the cells were blocked in S phase as revealed by a high labeling index of 86.5%, which is in good agreement with the frequency of S-PCC (91.4%). Removal of the drug by washing allowed the cells to progress through S phase indicating that the blocking effect of DAP was reversible.

Relationship Between Polyamine Levels and DNA Synthesis

These experiments were commenced by adding DAP to synchronized mitotic cells. DAP was found to have no effect on the completion of mitosis and the subsequent progression of these cells into G1 period as revealed by a rapid decrease in mitotic index from 98% to 12% within one hour. The effect of DAP treatment on the levels of the various polyamines is shown in Table 2.

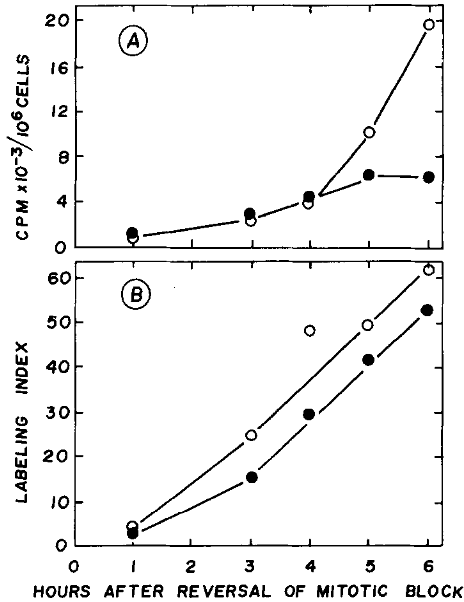

Fig. 1. Effect of DAP treatment on DNA synthesis.

A. Effect of DAP on the rate of incorporation of [^{3}H]-TdR into TCA-prepicitable fraction.

B. Effect of DAP on the rate of entry of G1 cells into S phase. ○, control; ●, DAP (13.5 mM) treatment.

This treatment had little or no effect on the levels of spermidine and spermine, whereas the amount of putrescine present in the treated cells had decreased to such a low level that it could not be detected by our method of analysis (less than 0.02 nmol). However, in the control the increase in the levels of putrescine coincides with the entry of cells into S phase. (Table 2, Fig. 1a).

The decrease in the putrescine level had resulted in a significant inhibition of incorporation of [^{3}H]-TdR into DNA (Fig. 1). On the contrary, the labeling index curve for the control differed only slightly from that of the treatment. These results suggest that the inhibition of putrescine biosynthesis stops DNA chain elongation but not initiation.

TABLE 3

EFFECT OF DAP TREATMENT ON POLYAMINE LEVELS

OF CHO CELLS DURING THEIR TRAVERSE

FROM S PHASE TO MITOSIS

Hours after reversal of TdR block	Control			DAP Treatment		
	n mole/4 x 10^6cells			n mole/4 x 10^6 cells		
	Putrescine	Spermidine	Spermine	Putrescine	Spermidine	Spermine
2	0.27	11.19	6.98	0*	9.3	6.54
4	0.23	9.82	6.49	0	8.10	6.53
6	0.27	8.98	6.78	0	6.02	3.68
8	0.31	8.80	5.95	0	5.16	4.50
10	1.25	10.06	8.52	0.04	4.98	4.18
12	1.39	9.87	8.29	0.02	6.98	4.89

*Less than the sensitivity of amino acid analyser (below 0.02 n moles).

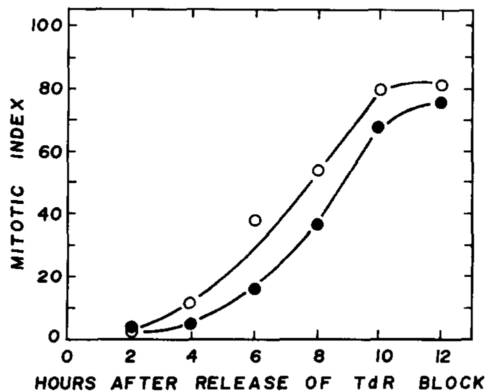

Fig. 2. Effect of DAP treatment on the traverse of S phase cells into mitosis. ○, control; ●, DAP treatment.

Relationship Between Polyamine Levels and Mitosis

CHO cells synchronized in S phase by the double TdR block method were treated with DAP and their entry to mitosis in the presence of Colcemid was followed by determining mitotic index and the levels of polyamines in samples taken at intervals of 2 hr. Untreated S phase cells served as control. The effect of DAP treatment on polyamine levels and the mitotic accumulation are shown in Table 3 and Fig. 2, respectively. An increase in the levels of putrescine was observed in the controls as the cells entered into mitosis. However, no such increase was seen in DAP-treated cells even though their rate of entry into mitosis was not significantly different from that of the controls. These data indicate that the inhibition of putrescine synthesis has no significant effect on the progression of cells from S phase to G2 and ultimately into mitosis.

DISCUSSION

The results of this study indicate that DAP treatment results in a decrease of putrescine levels (Table 2) and a concommitant inhibition of DNA synthesis in CHO cells (Fig. 1). The effect of DAP treatment was most pronounced on the amount of incorporation of [^{3}H]-TdR into DNA (Fig. 1a) but least on the progression of cells from G1 to S phase as indicated by the increase in labeling index (Fig. 1b). These data suggest that putrescine is essential for DNA chain elongation but not for its initiation. However, the levels of spermidine and spermine were not significantly affected by this treatment (Table 2).

Earlier studies with lymphocytes (9) and hepatoma cells (10) using different inhibitors suggest that among polyamines, spermidine may play an important part in DNA synthesis and cell division, although they have not completely ruled out the role of putrescine. The present study clearly indicates that putrescine is also essential for the completion of DNA replication. Russell and Stambrook (21) report that the activity of S-adenosylmethionine decarboxylase, the enzyme involved in the conversion of putrescine to spermidine,

increased in early S phase. Furthermore, spermidine biosynthesis has been shown to be essential for DNA chain elongation (9). In the light of these observations, the results of our study suggest that a decrease in the levels of putrescine induced by DAP treatment may lead to reduced rates of spermidine biosynthesis and consequently to the inhibition of DNA chain elongation.

Earlier observations (10,12) indicate that two peaks of putrescine biosynthesis occur; one during the late G1 period and the other just before mitosis. Heby et al. (12) suggest that the second peak of activity might be necessary for mitosis. However, we find that inhibition of putrescine biosynthesis in synchronized S phase cells did not prevent them from entering into mitosis (fig. 2 and Table 3). Therefore, we conclude that putrescine is necessary for DNA synthesis but may not be essential for mitosis and cell division.

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